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ORIGINAL PAPER

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Inhibition of Non-Homologous End Joining and integration of DNA upon transformation of *Rhizopus oryzae*

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Abstract Site-directed integration of DNA in the fungus *Rhizopus* has long been problematic because linearized plasmids used for transformation tend to replicate in high-molecular-weight concatenated structures, and rarely integrate into the chromosome. This work examines the methods that might interfere with the multimerization process, select against plasmids that had recircularized, and encourage strand invasion, hopefully leading to plasmid integration. In vitro methods were used to determine if the structure of the double-strand break had any effect on the ability to rejoin plasmid ends. In cell-free extracts, little difference in end-joining activity was found between linearized plasmids with 5′ overhangs, 3′ overhangs, or blunt ends. In addition, dephosphorylation of ends had no effect. Transformation of plasmids prepared in the same ways confirmed that they were easily religated in vivo, with almost all prototrophic isolates retaining autonomously replicated plasmids. It was possible to block religation by modifying the free ends of the linearized plasmids using oligonucleotide adapters which were blocked at the 3′-OH position and contained phosphorothioate nucleotides to make them nuclease-resistant. However, gene replacement, with repair of the auxotrophic mutation in the host chromosome, was the predominant event observed upon the transformation of these plasmids. The highest rates of integration were obtained with a plasmid containing a truncated, non-functional *pyrG* gene. Autonomous replication of this plasmid did not support prototrophic growth, but homologous recombination into the chromosome restored the function of the endogenous *pyrG* gene. All of the transformants obtained with this selective construct were found to have integrated the plasmid, with multicopy insertion being common.

Keywords Rhizopus · Transformation · Gene replacement · Integration

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Introduction

The filamentous fungus *Rhizopus* has long been recognized as an important microorganism that can be both beneficial and harmful. This fungus is well-known for the ability to produce industrial enzymes (e.g., glucoamylase, lipase), organic acids (e.g., lactate, fumarate), corticosteroids, and even fermented foods. However, it also is responsible for the majority of mucormycoses—infections that are difficult to treat in immunocompromised individuals. Furthermore, it is a plant pathogen that results in significant loss of agricultural commodities. Unfortunately, research with *Rhizopus* has been hampered by a lack of molecular techniques for the genetic manipulation of the fungus. Members of the Mucorales (e.g., *Rhizopus*, *Mucor*, *Absidia*, *Phycomyces*, *Rhizomucor*), an order of the class Zygomycetes, all share this drawback, owing to an incomplete understanding of the recombination and replication mechanisms that affect the fate of the introduced DNA. Unlike the case in most other fungi, DNA used for transformation rarely integrates into the chromosomes of Mucorales fungi, but is replicated autonomously in high-molecular-weight (HMW) concatenated structures (i.e., chains of linked monomers). It appears that autonomous replication in Mucorales does not require a defined origin of replication (Revuelta and Jayaram 1986; van Heeswijck 1986; Wostemeyer et al. 1987; Yanai et al. 1990; Benito et al. 1995; Skory 2002, 2004; Appel et al. 2004), and that non-specific regions of DNA can serve to initiate replication, as is common in

higher eukaryotes (Gilbert 2001). While such autonomous replication can occasionally be advantageous, site-specific integration is typically preferred and necessary for gene disruption. We are aware of only one example of a successful double cross-over gene knock-out in a Mucorales fungus (Navarro et al. 2001). However, Michielse et al. (2004) recently showed that introduction of DNA into *Rhizopus* by *Agrobacterium*-mediated transfer resulted in stable chromosomal integration in 100% of the isolates. In that study, 75% of the transformants had ectopically integrated part of the T-DNA, containing the homologous selection marker, at an undefined locus. The remaining isolates appeared to have undergone gene replacement of the auxotrophic *pyr4* marker and did not contain any of the flanking *Agrobacterium* T-DNA vector sequence.

Transformation, integration, and repair mechanisms have previously been described for *Rhizopus oryzae* (Skory 2002, 2004). It has been shown that introducing a double-strand break (DSB) within the homologous regions of the plasmid DNA used for transformation can significantly improve the likelihood of integration, up to 20% of the total isolates. However, the majority of transformants will still have plasmid replicating autonomously in HMW concatenated structures. It is thought that this occurs because linearized DNA is religated—before integration can occur—by repair mechanisms collectively known as non-homologous end joining or NHEJ (Lieber 1999; Frank-Vaillant and Marcand 2002; Ray and Langer 2002). In yeast, NHEJ requires that Ku80-Ku70 heterodimers bind to DNA ends and translocate inward in an ATP-independent manner. DNA-dependent protein kinase (DNA-PK) then forms a complex that allows DNA ligase IV and its cofactor XRCC4 to ligate the ends. The ligation step needs only a few basepairs of homology, and occasionally leads to mispaired or misaligned strands, which results in changes in the rejoined site (Sandoval and Labhart 2002). Significant homology on either side of the DSB, such as the presence of a direct repeat, will typically favor single-strand annealing or SSA over NHEJ (Lin et al. 1984, 1985). This effect, which has been demonstrated in *Rhizopus*, is believed to be due to homologous recombination between the duplicated sequences, resulting in the elimination of one of the direct repeats (Skory 2002). This can occur by 5′ to 3′ DNA strand resection of each end at the DSB to expose homologous sequence, or by removal of flaps formed when each of the direct repeats anneal (Paques and Haber 1999; Heyer et al. 2003; Prado and Aguilera 2003). Regardless of whether religation of the DSB occurs by NHEJ or SSA, these repair mechanisms prevent integration by mending the free DNA ends before cross-over mechanisms can be initiated.

We have previously attempted to devise methods that select against plasmids that have been repaired by NHEJ (Skory 2004). One approach involved the introduction of a frameshift mutation into the selectable *pyrG* gene that encodes the *R. oryzae* orotidine 5′ monophosphate decarboxylase. It was hypothesized that religation of the plasmid would not allow prototrophic growth because of the mutation present in the plasmid. However, growth should be possible if homologous integration and cross-over occurs between the *pyrG* genes in the region flanked by the plasmid mutation and the genomic *pyrG* mutation responsible for the auxotrophic phenotype. This type of additive integration (Hinnen et al. 1978) would result in duplication of the *pyrG*, with one non-functional copy containing both mutations and the second copy being fully functional (if no additional mutations were introduced during the cross-over). While this recombination event was detected, the majority of transformants contained autonomously replicating plasmids in concatemer arrangements. Sequence analysis showed that, in these cases, prototrophic growth was restored by repairing the non-functional *pyrG* sequence in the plasmid, while the genomic copy of *pyrG* remained unaltered. It is thought that this type of gene conversion occurs via recombination pathways involving synthesis-dependent strand annealing (SDSA) or break-induced replication (BIR) mechanisms, which use the genomic homologue as a template to synthesize new template (Paques and Haber 1999; Lewis and Resnick 2000; Heyer et al. 2003; Prado et al. 2003). Unlike additive integration or gene replacement events, these gene conversion events do not involve cross-over or reciprocal exchange of DNA.

Integration of the plasmid, on the other hand, must proceed by double-strand break repair (DSBR) recombination, which is initiated by processing the ends of the break to yield 3′ single-stranded tails (Szostak et al. 1983; Sun et al. 1991). The 3′ end of one of the strands invades the unbroken homologue, or chromosomal template in this particular case, to form a displacement (D-) loop (Paques and Haber 1999). Both 3′ ends of the DSB can then prime new DNA synthesis using the chromosomal strand as template. A double Holliday junction (DHJ) is then formed when the invading strand joins the other arm or 5′ end of the DSB (Holliday 1964; Collins and Newlon 1994; Schwacha and Kleckner 1994). Resolution of the HJ occurs by cleavage through either plane, which results in either additive integration of the plasmid or its release (Orr-Weaver and Szostak 1983; Ira et al. 2003; Merker et al. 2003; Prado and Aguilera 2003). SDSA differs from DSBR in that capture of the 5′ end of the DSB does not occur and no DHJs are formed. Instead, the invading strand is displaced and reanneals with the original strands. BIR is similar to SDSA, but involves only one end of the DSB, such as the repair of a broken chromosome (Kraus et al. 2001).

Because the NHEJ and homologous recombination pathways appear to compete with one another (Ray and Langer 2000; Richardson and Jasin 2000; Saintigny et al. 2001; Frank-Vaillant and Marcand 2002; Kooistra et al. 2004), one of the goals of this work was to investigate further methods that might interfere with NHEJ and encourage 3′ strand invasion that would hopefully

lead to the formation of HJs. In vitro methods were used to determine if the structure of the DSB had any effect on the ability to rejoin plasmid ends. In addition, several of these constructs were transformed into *R. oryzae* to test the efficiency of integration. While the incidence of NHEJ could be reduced, there was no corresponding improvement in the efficiency of integration. Therefore, methods were designed to select against plasmids that had recircularized through NHEJ, by using a plasmid containing a non-functional *pyrG* gene for transformation. In this case, instead of introducing a mutation into the selectable marker, the *pyrG* gene was truncated so that only homologous additive integration of the plasmid would restore function.

Materials and methods

Fungal transformations

Rhizopus oryzae (syn. *R. arrhizus*) NRRL 395 was the source strain for the OMP decarboxylase auxotroph *R. oryzae* PYR-17 (*pyr181*) used in this study. This strain has been described in detail and contains a G to A transversion 181 bp downstream of the start codon, at the 5′ end of a splice junction (Skory 2002). Plasmid pPyr225 contains the *R. oryzae pyrG* gene and is capable of restoring prototrophic growth when transformed into *R. oryzae* PYR-17. Transformation of *R. oryzae* PYR-17 was carried out by microprojectile particle bombardment (BioRad, Hercules, CA, USA) as previously described (Skory 2002). Ungerminated spores were transformed directly on RZ minimal glucose medium plates, since no recovery time for auxotrophic selection was required. Approximately 5–7 days following bombardment, spores were collected and diluted in sterile water to obtain single-spore isolates. Only one isolate per plate was used for further analysis in order to avoid multiple progeny originating from the same transformation event. The plasmid DNA used for transformation was either the covalently closed circular form or was linearized by digestion with restriction endonuclease (RE). Linear plasmid was always isolated by agarose gel electrophoresis and purified using a Qiaquick Gel Extraction kit (Qiagen, Valencia, Calif.) to eliminate any contaminating uncut DNA. Modifications to the linearized plasmid are described below.

In vitro analysis of end-joining

Cell-free protein extracts used for end-joining assays were prepared from *R. oryzae* grown for 18 h in RZ minimal glucose medium (Skory 2002). The harvested mycelium was immediately frozen in liquid nitrogen and pulverized using a ball mill apparatus (Retsch, Haan, Germany). The frozen powdered mycelium was slowly dissolved in ice-cold EJ Buffer (50 mM HEPES pH 7.8, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg leupeptin/ml, 1 μg pepstatin A/ml) and then centrifuged at 250,000× *g* for 2.5 h at 4°C. The cleared lysate was loaded into a dialysis bag (cutoff MW 3,350) and dialyzed against four changes of excess EJ buffer at 4°C for 2 h, before aliquoting and freezing with liquid nitrogen at a concentration of 6.4 mg protein/ml.

End-joining assays were performed with plasmids containing several types of DSBs. Plasmid pBluescript KS(-) was digested with either *Bam*HI, *Pst*I or *Eco*RV to generate each of the three typical end configurations (i.e., 5′ overhang, 3′ overhang, and blunt end, respectively) obtained with REs. Some of the digested DNA from each reaction was further modified by removal of the 5′ terminal phosphate with calf alkaline phosphatase (CIP; New England Biolabs, Beverly, MA, USA), added at ten times the concentration recommended by the manufacturer. The reaction was terminated and the plasmid was purified by gel electrophoresis. The efficiency of the dephosphorylation step was confirmed by the fact that no ampicillin-resistant *Escherichia coli* isolates with plasmid could be obtained after the plasmid was incubated with ligase. Plasmid pPyr225, which was linearized with *Sty*I and modified with phosphorothioate adapters, is described below. Reactions (10 μl) containing 8 μl of cell-free extract (see above), 25 ng of plasmid DNA, 1 mM ATP and 1 mM $MgCl_2$ were allowed to proceed for a period of 0–3 h at 30°C. The reaction was stopped by the addition of 90 μl of termination buffer (50 mM TRIS-Cl pH 8, 0.3 M sodium acetate, 10 mM EDTA, 0.5% SDS). Samples were extracted with phenol chloroform and precipitated with ethanol. DNA resuspended in TE was then subjected to agarose gel electrophoresis in the absence of ethidium bromide, transferred to nylon membranes by alkaline transfer, and detected by Southern hybridization as described below.

Modification of DSBs in linearized plasmids used for transformation

Plasmid pPyr225 was modified in several different ways to vary the structure of the DSB created by RE digestion. Digestion with *Sty*I results in linearization of pPyr225 within the *pyrG* gene and creates 5′ overhangs at the site of the DSB. Digestion with *Dra*III, which also cuts within the *pyrG* gene, was used to create 3′ overhangs. However, this was not possible in plasmid pPyr225, since other *Dra*III sites were present in the pBluescript vector. Therefore, the 2.25-kb *pyrG*-containing fragment was transferred to pUC18 following digestion with *Bam*HI and *Kpn*I. The resulting 4.99-kb plasmid is referred to as pPyr89B18. Removal of the 5′ terminal phosphate from both linearized plasmids was performed as described above.

Plasmid ends were also modified by exposing long stretches of 3′ single-stranded overhangs by limited digestion with Lambda exonuclease (New England

Biolabs), which catalyzes the removal of 5′ mononucleotides in the 5′ to 3′ direction. Nuclease digestion of *Sty*I-digested pPyr225 (4 U of exonuclease per pmol of linearized plasmid) was performed in the manufacturer's recommended buffer for 10 min at 30°C. The reaction was stopped using the Qiaquick PCR purification kit. Alkaline gel electrophoresis (Sambrook et al. 1989) confirmed that these conditions resulted in only partial (< 200 nt) resection of the 5′ end of the DSB.

Free ends of the plasmid created by *Sty*I digestion of pPyr225 were also blocked or capped with adapters that incorporated nuclease-resistant phosphorothioate nucleotides (Verma and Eckstein 1998) to minimize the 5′–3′ processing typically required for recombination of the DSB. This was accomplished by first creating two sets of adapters for ligation onto each end of the *Sty*I break. Adapters were made by annealing oligonucleotides in TES (10 mM TRIS-HCl pH 8, 1 mM EDTA, 50 mM NaCl). Adapter Set-A consisted of the sequences 5′-caagCAGAGAGG*G*G* 3′ and 5′-cttgCAGAGAGG*G*G*-3′. Nucleotides shown in lower case represent single-stranded *Sty*I-compatible overhangs and nucleotides in upper case are double stranded. Phosphorothioate nucleotides (Integrated DNA Technologies, Coralville, IA, USA) were included in both the sense and antisense strands and are indicated by the asterisks. In addition to these blocking adapters, an identical Set-B was prepared that contained a 3′ amino modifier (C7; Integrated DNA Technologies) in place of the terminal 3′-OH group, which was used to block potential priming by polymerase enzymes (Zhang and Li 2003; Gale and Tafoya 2004). A 500-fold excess of each adapter set was ligated overnight at room temperature to *Sty*I-linearized pPyr225 in a reaction that also contained *Sty*I enzyme to digest re-circularized plasmid. The linear plasmid was gel-purified and then subjected to another overnight self-ligation and gel purification to remove any fragments that were not blocked with the modified adapters. The efficiency of blocking was assessed by incubating each of the final purified fragments for 1 h at room temperature with T4 DNA ligase. The nuclease resistance of the modified *pyrG* fragments was tested by exposing each preparation to Lambda exonuclease (at a concentration of 200 U per pmol of DNA) for 1 h at 37°C. *Sty*I-linearized pPyr225 served as control for each of these reactions. We also tested an additional set of phosphorothioate-containing adapters (5′-C*C*C*CTCTCTg/c-3′) with the amino modifier C7 in place of the 3′-OH. These adapters were designed for ligation to the *Ahd*1-linearized plasmid. This restriction site is located outside of the selectable *pyr*G marker within the pBluescript vector sequence.

Construction of pPyrG-FH plasmid

Plasmid pPyr225 was modified to contain only the upstream promoter sequence and the 5′ half of the *pyrG* coding region. This was accomplished by digesting pPyr225 with *Sty*I and *Bam*HI, which cleave within the *pyrG* coding sequence and downstream in the vector polylinker, respectively. The downstream region of the *pyrG* was removed by gel electrophoresis, and the remaining 4.7-kb plasmid was recircularized after blunting with T4 DNA polymerase. The plasmid containing the non-functional 5′ half of the *pyrG* gene, called pPyrG-FH (for Front Half), was linearized with *Mfe*I prior to transformation.

A 3.3-kb *Xho*I- *Cla*I fragment of the *Rhizopus ldhA* gene from plasmid pLdhA48XI-A (Skory 2005) was cloned into the corresponding sites in pPyrG-FH, allowing us to test for the integration of an unselected insert using plasmid pPyrG-FH. The resulting plasmid, pPyrG-FH/Ldh, was transformed as previously described.

Molecular analyses

Southern analyses were performed using the Genius system (Boehringer Mannheim, Indianapolis, IN, USA) according to the manufacturer's recommendations. Digoxigenin labeled Lambda DNA, cleaved with *Hin*dIII, served as a molecular weight marker on Southern gels. The probe for end-joining assays [an 825-bp region of the *β*-lactamase gene of the pBluescript KS(-) vector was amplified by PCR with the primers 5′-CACGCTCACCGGCTCCAGATTTA-3′ and 5′-ATGTGCGCGGAACCCCTATTTGT-3′. The probe used for analyses of transformants was an internal 848-bp region of the *pyrG* gene obtained by PCR with the primers 5′-ATAGCGAGCGTGCCAAACAAC-3′ and 5′-TTCAAGATATGCGTCCCAACCA-3′. DNA from transformants was digested with *Bst*BI, which does not cut pPyr225 or pPyr89B18. Replicating plasmids are detected by Southern analysis as HMW multimeric structures. Amplification of the upstream copy of the *pyrG* gene from transformants which had integrated the plasmid pPyrG-FH was performed with primers P1 (5′-CGCCCTTACCTATCGTATCTCA-3′), which anneals upstream of the genomic *pyrG* promoter region not present in the plasmid, and P2 (5′-CCGATTTCGGCCTATTGGTTA-3′), which anneals to the pBluescript II vector sequence. Amplification of the downstream copy was performed with primers P3a (5′-ACCGAGCGCAGCGAGTCAGTG-3′), which anneals to the pBluescript II vector sequence and P4 (5′-CCGCGGTGAAGATGAGGATGAGA-3′), which anneals downstream of the genomic *pyrG* terminator region not present in the plasmid. Amplification of the downstream duplicated *pyrG* sequence from isolates that had integrated plasmid pPyrG-FH/Ldh was similar, except that primer P3b (5′-GTTCAACGGGCTCAGCAGGTCTAC-3′), which anneals to the *ldhA* fragment on the plasmid, was used in place of P3a.

Results

In vitro analysis of end-joining

Several different constructs with varied overhangs on the free DNA ends were tested for end-joining using cell-free extracts prepared from *R. oryzae*. Such an activity was first demonstrated using only pBluescript vector DNA digested with *Bam*HI, which results in 4-nt 5′ overhangs. The reaction products were analyzed every 30 min over a period of 3 h (Fig. 1). A reaction immediately treated with termination buffer served as a control, and a distinct monomeric (M) 3.0-kb band with almost no degradation or uncut plasmid present can be seen at this point. Dimers (D) of the plasmid and degradation products are clearly detected within 30 min of initiating the reaction, followed by the appearance of trimers (T) which are faintly detected after 1 h. The levels of dimers and trimers seem to decrease after 1 h, and HMW plasmid that fails to migrate into the gel appears abruptly at 1.5 h and remains in the loading well. In addition, the smear of DNA extending from 3.0-kb to the loading well becomes most prominent at this time. After 1.5 h, amounts of ligated plasmid DNA larger than 3.0 kb appear to decrease. However, the amount of degradation products does not seem to change significantly from 0.5 to 3 h. It is also interesting to note that most of the degradation products are of distinct sizes, suggesting that cleavage results from the action of sequence-specific nucleases. Reaction time of 1.5–2.5 h seemed to give optimal end-joining with minimal degradation. We therefore choose the 2-h time point for analyzing plasmids with modified DNA ends.

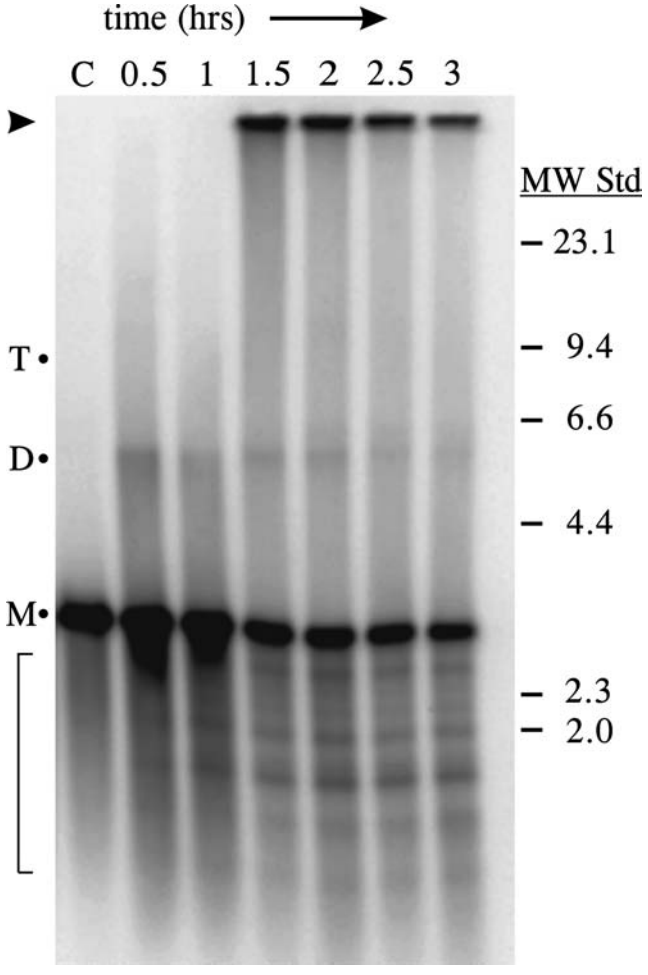

Fig. 1 In vitro end-joining assay. Plasmid pBluescript digested with *Bam*HI was incubated with *Rhizopus* extracts in reaction buffer, the reactions were terminated at the indicated times, and processed for Southern hybridization after gel electrophoresis, using pBluescript as the probe. The reaction shown in lane C was terminated immediately after mixing all components, and was used as a control. Lanes 1–6 represent reactions allowed to proceed for between 0.5 and 3 h, as indicated. The *arrow* at the *top* of the blot shows the relative location of the loading wells. *M*, *D*, and *T* indicate monomeric, dimeric, and trimeric plasmids, respectively. The *bracket* on the *left* marks the region containing degradation products. The location and size (in kb) of *Hin*dIII fragments of Lambda DNA used as MW standard are shown to the *right* of the blot

Plasmids digested with *Pst*I (3′ overhang) or *Eco*RV (blunt-ended, no overhang) did not seem to differ significantly in their susceptibility to end-joining from the *Bam*HI-digested molecules. In each case, multimers and HMW DNA were formed. Furthermore, dephosphorylation of all three linearized vectors made almost no difference to the levels of multimers formed (data not shown).

Plasmid pPyr225 linearized with *Sty*I was modified by ligating phosphorothioate containing adapters onto the 5′ overhangs. The efficiency of this modification was first tested prior to using DNA for end-joining assays or transformations. Neither of the plasmids containing primer set A or B showed any indication of ligation after incubation with T4 DNA ligase, while control plasmid digested with *Sty*I was easily ligated (Fig. 2). Furthermore, both modified plasmids containing nuclease-resistant phosphorothioate nucleotides showed resistance to Lambda exonuclease compared to the control plasmid (which was almost completely degraded). However, the results of in vitro analysis of end-joining with *Rhizopus* protein extracts were not as clear-cut.

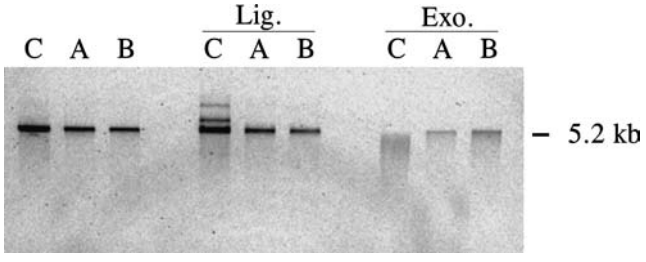

Fig. 2 Agarose gel electrophoresis of *Sty*I-digested pPyr225 ligated to phosphorothioate-containing adapters. Plasmid pPyr225 digested with *Sty*I served as the control (*C*). Linearized plasmid modified with Adapter Sets A and B are labeled in each lane. The first *three lanes* show each of the 5.2-kb plasmids after linearization and modification with adapters. Plasmids subjected to self ligation with T4 DNA ligase are shown in the next set (*Lig*), while plasmids treated with Lambda exonuclease are in the *last three lanes* (*Exo*)

Both sets of modified plasmids yielded almost the same amount of ligated products as the *Sty*I digested control, although plasmid-containing adapter Set-B made with the 3′ amino modifier C7 seemed to give rise to slightly less high MW DNA in wells compared with control (data not shown).

Transformation of *R. oryzae* with modified plasmids

Transformation of *Rhizopus* using plasmid pPyr225 digested with *Sty*I has previously been described (Skory 2002, 2004) and was used in this study mainly to confirm the efficiency of transformation. As expected, most of the transformants obtained with this linear plasmid were found to have high MW plasmid replicating autonomously in a concatenated structure. Homologous integration of the plasmid into the *pyrG* locus occurred in less than 20% of the isolates, with only one isolate having a single additive integration of the plasmid (Table 1). Altering the configuration of the DSB by digestion of plasmid pPyr89B18 with *Dra*III, which leaves a 3′ overhang, did not improve the efficiency of integration. Southern analysis using *Bst*BI digested DNA from ten transformants showed that 90% of isolates had autonomously replicating plasmids (Table 1 and Fig. 3). Since *Bst*BI does not cut plasmid pPyr89B18, replicating plasmids are detected by Southern analysis as HMW multimeric structures. These plasmids are often present in high copy numbers (40–70 per nucleus), as seen in selected transformants on this Southern blot. No isolates with integrated plasmid were found among 10 transformants obtained with *Dra*III-digested pPyr89B18. One of the isolates shown in Fig. 3 (lane 2) had no detectable plasmid and is believed to be the result of a DSBR-mediated gene replacement event. Dephosphorylation of plasmid pPyr225 digested with *Sty*I and plasmid pPyr89B18 digested with *Dra*III did not seem to affect the ability of transformants to recircularize plasmids, since all 20 of the isolates had replicating plasmid with no evidence of integration (Table 1). *Sty*I-digested plasmid pPyr225 was treated with Lambda exonuclease to expose 100- to 200-nt stretches of 3′ single-stranded overhangs. Southern analysis of ten transformants obtained with this modified plasmid revealed that none of the isolates showed any evidence of integration or gene replacement (Table 1 and Fig. 3). All of the isolates had autonomously replicating plasmids as previously seen.

Transformations with *Sty*I-digested plasmid pPyr225 ligated to phosphorothioate-containing adapters yielded interesting results regarding the requirement for the 3′-OH on the free DNA end. None of ten transformants obtained with plasmid bearing Set-A adapters, which contain a functional 3′-OH, showed any evidence of integration (Table 1). All of the isolates showed HMW replicating plasmid upon Southern analysis of *Bst*BI-digested DNA, as shown for selected transformants in Fig. 4 (lanes 1–4). However, blocking of the 3′-OH with the amino modifier C7 (Adapter Set B) resulted in repair

Table 1 Analysis of *Rhizopus* transformants obtained with modified plasmids

Repair event[a]	Plasmid (modification)[b]										
	pPyr225 (StyI)	pPyr89B18 (DraIII)	pPyr225 (StyI/CAP)	pPyr89B18 (DraIII/CAP)	pPyr225 (StyI/Exo)	pPyr225 (StyI/AdptA)	pPyr225 (StyI/AdptB)	pPyr225 (AhdI/Adpt)	pPyrG-FH	pPyrG-FH/Ldh
NHEJ	8	9	10	10	10	10	2	10	–	–
Additive integration (DSBR)	1	–	–	–	–	–	–	–	7[c]	2
Gene replacement (DSBR)	1	1	–	–	–	–	8	–	–	2

[a] HMW multimeric plasmids detected by Southern analysis are presumed to result from NHEJ of the linearized input plasmid; integration of plasmid is considered to be due to repair by DSBR-mediated mechanisms, which result in either additive integration or gene replacement

[b] See text for details

[c] Single-copy integration, four isolates; double-copy integration, two isolates; triple-copy integration, one isolate

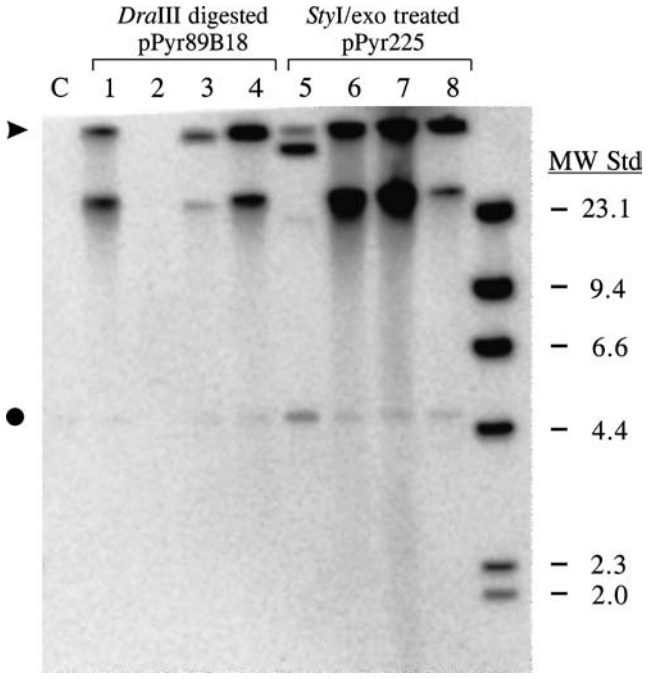

Fig. 3 Southern hybridization analysis with *pyrG* of *Bst*B1-digested DNA obtained from selected isolates transformed with plasmid DNA containing 3′ overhangs. Untransformed *Rhizopus oryzae* DNA served as a control (*lane C*). *Lanes 1–4*, DNA from selected transformants obtained with *Dra*III-digested pPyr89B18; *lanes 5–8*, DNA from selected transformants obtained from *Sty*I-digested pPyr225 treated with Lambda exonuclease to expose 3′ single-stranded DNA. The *arrow* at the *top* of the blot shows the relative location of the loading wells. The native *pyrG* gene appears as a 4.7-kb band in all sample lanes and is marked with a *filled circle*. The low intensity of these bands is due to the fact that the total signal strength on the blot had to be reduced to avoid saturation by the replicating plasmids that are present in multiple copies. Labeled *Hind*III fragments of Lambda DNA were used as the MW standard, and their sizes are indicated in kb to the right of the blot

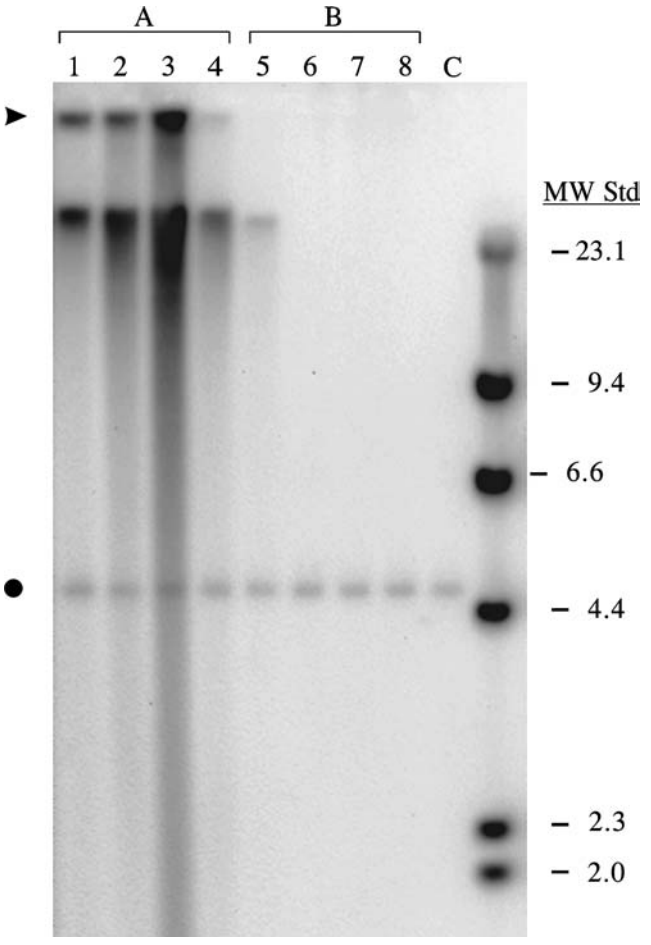

Fig. 4 Southern hybridization analysis with *pyrG* of *Bst*B1-digested DNA obtained from selected isolates transformed with *Sty*I-digested pPyr225 DNA modified with phosphorothioate-containing adapters. Untransformed *R. oryzae* DNA served as a control (*lane C*). *Lanes 1–4* DNA from selected transformants obtained with plasmid modified with Adapter Set-A, which contains a free 3′-OH at the ends; lanes 5–8, DNA from selected transformants obtained with plasmid modified with Adapter Set-B, which contains an amino modifier C7 in place of the 3′-OH. The *arrow* at the *top* of the blot shows the relative location of the loading wells. The native *pyrG* gene appears as 4.7-kb band and is marked with a *filled circle*. Labeled *Hind*III fragments of Lambda DNA were used as the MW standard, and their sizes are shown in kb to the *right* of the blot

of the genomic mutation in 80% of the transformants, as shown for selected transformants in Fig. 4 (lanes 6–8). Only two of the 10 transformants retained autonomously replicating plasmids in this case, as shown in lane 5. We also used the same strategy of blocking the 3′-OH with phosphorothioate-containing adapters with the amino modifier C7, but linearized and capped the plasmid approximately in the middle of the vector sequence at the *Ahd*1 site. In this case, all ten of the isolates obtained had autonomously replicating plasmids (Table 1). Further analysis by Southern hybridization using enzymes that cut within the plasmid revealed that 1–2 kb of vector sequence in the vicinity of the *Ahd*1 site had been deleted in most of the plasmids detected in transformants (data not shown).

Transformation of *R. oryzae* with the 5′ half of the *pyrG* gene

Plasmid pPyrG-FH was designed so that functionality of the *pyrG* gene could only be restored by homologous integration. The plasmid was linearized with *Mfe*I prior to transformation to facilitate integration by DSBR-dependent mechanisms. Transformation efficiency, i.e., restoration of prototrophic growth, with this construct was difficult to measure as is typical with biolistic methods but was roughly estimated to be only 1–2 transformants/μg DNA—or approximately 20-fold less than that obtained with plasmid pPyr225. However, Southern analysis of DNA from three transformants revealed that in 100% of the isolates plasmid pPyrG-FH had integrated into the chromosome (Fig. 5). Digestion of DNA with *Bst*BI, which does not cut the plasmid, showed that transformants had 1, 2, and 3 copies of the plasmid integrated into the *pyrG* locus, as revealed by

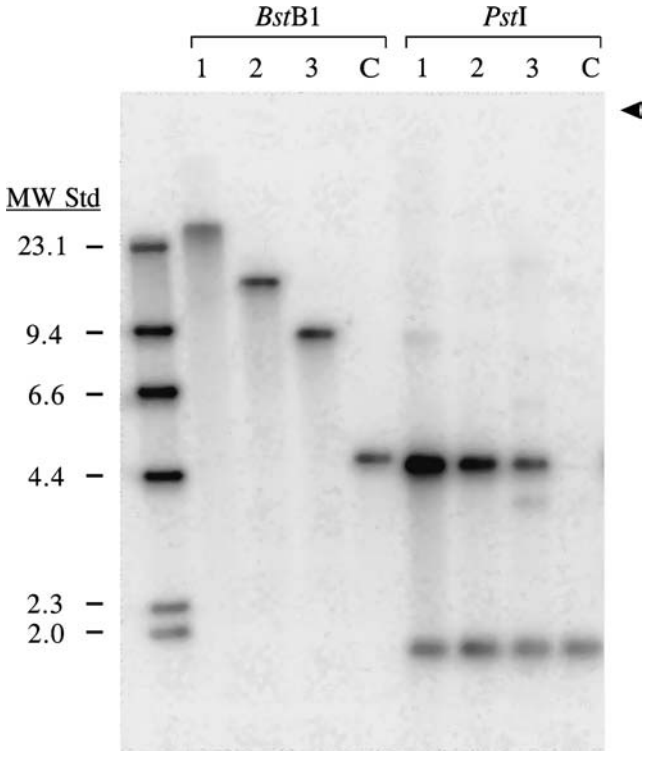

Fig. 5 Southern hybridization analysis with *pyrG* of DNA obtained from isolates transformed with pPyrG-FH. Untransformed *R. oryzae* DNA served as a control (*lane C*), while transformants are labeled as isolates 1–3. DNA was digested with either *Bst*B1 or *Pst*I. The *arrow* at the *top* of the blot shows the relative location of the loading wells. Labeled *Hind*III fragments of Lambda DNA were used as the MW standard, and their sizes are indicated in kb to the *right* of the blot

the increased size of hybridizing bands when compared to the 4.7-kb *pyrG* band of the untransformed control. DNA used for Southern analysis was also digested with *Pst*I, which cuts once within pPyrG-FH. All of the transformants had 4.5-kb bands that are associated with the integrated plasmid (Fig. 6) and increase in intensity with copy number. The 1.7-kb band that represents the full length *pyrG* is present in all isolates. Sequence analysis of each of the duplicated *pyrG* genes confirms that integration occurred as expected and that functionality of the gene was restored in the second copy. We found that uncut plasmid integrated in the same manner, and that linearization did not significantly alter the transformation efficiency. All the isolates obtained with plasmid pPyrG-FH using these different transformation methods were found to show homologous additive integration at the *pyrG* locus. Interestingly, transformation of pPyrG-FH/Ldh resulted in a shift toward gene replacement, although it is not known if secure conclusions can be drawn in view of the small numbers of isolates examined (Table 1). Sequence analysis of the flanking *pyrG* locus confirmed that integration of pPyrG-FH/Ldh had occurred in the predicted manner and that this approach can be used successfully for targeted integration of an unselected DNA of interest.

Discussion

This work had the overall objectives of trying to inhibit NHEJ, improve the efficiency of strand invasion to facilitate DSBR, and develop methods that would select against plasmids that had recircularized. Because NHEJ and DSBR are considered competing pathways, it was hypothesized that inhibition of NHEJ would allow homologous integration by DSBR-dependent mechanisms to occur more readily. Recent work by Kooistra et al. (2004) has demonstrated that deletion of genes involved in NHEJ mechanisms in *Kluyveromyces lactis* resulted in a higher gene-targeting efficiency.

Efforts directed toward the first two goals centered primarily on the structure of the DSB. Methods of

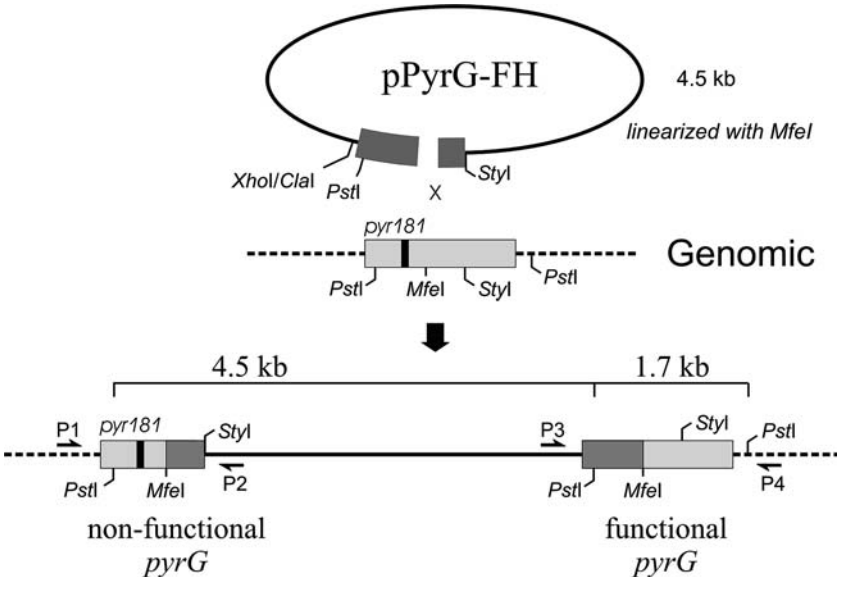

Fig. 6 Proposed mode of integration of pPyrG-FH plasmid. Plasmid pPyrG-FH, linearized with *Mfe*I, integrates at the homologous position in the genomic *pyrG* gene, just downstream of the *pyr181* mutation. This crossover results in the truncated *pyrG* fragment being associated with the genomic mutation, while second copy of the *pyrG* is restored to functionality. Primers P1–P4 were used to amplify each of the duplicated *pyrG* genes in the flanking integration locus

inhibiting NHEJ were initially tested by using in vitro end-joining assays to test the efficiency of religation of linearized plasmids in cell-free extracts. This type of assay has been very successful in studying the mechanisms of NHEJ in complex eukaryotic systems (Baumann and West 1998; Labhart 1999; Zhong et al. 2002). The effects of different types of DSB were tested by creating different types of overhangs by digestion with common Type II REs.

The first assay to determine optimal end-joining conditions was performed with plasmid linearized with *Bam*HI to generate 5′ overhangs (Fig. 1). The appearance of dimers became quite obvious within 30 min of initiating the reaction and trimers were faintly detectable after 1 h. Even though the concentration of multimers decreased after 1 h, the amount of HMW DNA that remained at the top of the gel became significant only after 1.5 h. Such HMW DNA resulting from this type of end-joining assay is unusual. It is more typical to obtain predominantly short multimers. The absence of obvious covalently closed monomers and multimers could be a result of varying degrees of supercoiling and thus mobility, since gel electrophoresis was performed in the absence of ethidium bromide. We chose 2 h as the optimal termination time for all further assays, since both multimers and HMW DNA were present at this point. In addition, the unusual HMW DNA obtained in this assay appeared better to reflect the concatenated plasmids found in the majority of *Rhizopus* transformants. The presence of degraded fragments in the assay is common and indicates a depletion of components necessary for NHEJ in the cell-free extract. Work by Blanco et al. (2004) demonstrated that these degradation products can be avoided by supplementing extracts with purified histone H1, but we decided that adding mammalian histone to our assays might introduce artifactual results.

Using plasmid linearized with restriction enzymes that generate 3′ overhangs and blunt ends at the DSB did not change the end-joining efficiency much when compared to that seen with the 5′ overhang. Indeed, even removing the 5′ phosphate from the DSB termini made almost no difference to end-joining. This is typical of NHEJ in mammalian systems, where even non-complementary ends are efficiently joined. Studies with lymphoblastoid cell lines show that polynucleotide kinases in the cell-free extracts efficiently phosphorylate the 5′-OH and allow end-joining to occur (Chappell et al. 2002). Still, we felt that it was important to test even simple overhangs that are capable of pairing interactions, since no other similar studies have been performed in filamentous fungi. The results of end-joining assays with linearized plasmid ligated to phosphorothioate-containing adapters did not suggest that the ability to ligate these plasmids by NHEJ was impaired.

The results of the in vitro assay indicated that none of the techniques we tried were very efficient at inhibiting NHEJ. However, this assay only tests for the ability to ligate DSBs, and does not provide any information about whether the integrity of the sequence at the DSB has been altered as a result of the repair. We were particularly interested in whether 3′ overhangs would be more likely to invade the genomic DNA, which is a prerequisite for initiating DSBR and integration. Therefore, we used plasmids containing DSBs with 3′ or 5′ overhangs for transformation of *Rhizopus*, and found that both performed equally well in restoring prototrophy. The efficiency of integration did not vary significantly and dephosphorylation made little difference. Even exposing more of the 3′ end by processive removal of the 5′ terminus by treatment with Lambda exonuclease did not prevent end-joining: all of the transformants retained plasmids that replicated autonomously in the typical HMW concatenated structures.

The only modification that had any significant effect on the competing NHEJ and recombination pathways was the ligation of phosphorothiate-containing adapters to the DSB termini. Figure 2 clearly shows that ligation by T4 ligase of both sets of modified fragments is prevented. However, this really only demonstrates the efficiency of constructing the modified fragment, since the lack of 5′-phosphate on the adapters is itself sufficient to block ligation by T4 ligase. The effects of treatment with Lambda exonuclease shown in the same figure demonstrate that the incorporation of the phosphorothioate nucleotides does not prevent nuclease action, but renders these fragments more resistant to degradation by this enzyme. This was important, because we wanted to minimize the likelihood of 5′ to 3′ resection of the linearized fragment upon transformation into the *Rhizopus*. The adapter sets each added an additional 10 nt that would force the *pyrG* out of frame if simply religated by NHEJ. Plasmids repaired in this manner would not restore prototrophy because the product of the *pyrG* gene would not be functional. If resection of the 5′ termini could occur, this would expose homologous sequence of the *Sty*I overhangs and allow SSA to repair the plasmid and restore *pyrG* function.

We believe that this is probably what happened with plasmid modified with Adapter Set A, which had a functional 3′-OH on each adapter. All of the isolates obtained from this experiment had autonomously replicating plasmid in HMW concatenated form. However, when the 3′-OH was blocked with amino modifier C7, 80% of the isolates had no detectable plasmid, suggesting that they had repaired the genomic *pyr181* mutation by DSBR-mediated gene replacement. Blocking of the 3′-OH with the modifier group would be likely to interfere with NHEJ by preventing ligation of the DSB. However, it is not obvious how this would interfere with repair by SSA, since the excess unpaired overhang could be removed by exo- or endonucleases. Furthermore, one might assume that if the blocked 3′-OH inhibits ligation by NHEJ, then it will probably interfere with the subsequent capture and ligation of the 5′ strand to form the DHJ. We were surprised to

see that none of the plasmids had integrated in these recombination events. The number of isolates tested was small, so it is difficult to draw any conclusions regarding the preference for cleaving the HJ in either plane (Orr-Weaver and Szostak 1983; Ira et al. 2003; Merker et al. 2003; Prado and Aguilera 2003), which ultimately would determine whether cross-over leads to integration of the plasmid, repair of the chromosomal *pyrG* mutation, or repair of the plasmid *pyrG*. Many additional recombination events probably occurred, but auxotrophic selection would have allowed the recovery only of outcomes leading to a functional gene. This was confirmed when adapters modified with the blocked 3′-OH were used at a DSB outside of the plasmid *pyrG* gene. This would remove selective pressure to ensure precise repair of this DSB, and this factor probably explains why extensive deletions occurred in this experiment. Exonuclease activity and resection of the 5′ strand could have easily proceeded until regions of homology were fortuitously exposed to allow repair by pathways such as SSA. While the modified plasmids containing the phosphorothioate adapters with the blocked 3′-OH helped to prevent NHEJ and presumably allow DSBR to occur, the efficiency of additive plasmid integration was still low. Therefore, we decided to develop methods that allowed both pathways to occur, but selected only for transformants with the desired additive plasmid integration.

With this intention, we designed a selection method that would allow prototrophic growth only if additive integration or gene replacement of the mutated *pyrG* had occurred. This was accomplished by following a strategy similar to the one used in previous studies where a frameshift mutation was introduced into the *pyrG* gene in the plasmid, so that autonomously replicating plasmids should not be able to provide functional orotodine monophosphate decarboxylase activity. However, DNA repair events, such as SDSA and BIR, were able to restore the frameshift mutation to functionality by gene conversion events. In the present study, such gene conversion events were prevented by creating plasmid pPyrG-FH, which contains the upstream promoter sequence and only the front (5′) half of the *pyrG* coding region. Autonomous replication of this plasmid should not be capable of restoring prototrophy by itself. However, homologous additive integration into the mutated genomic copy of the *pyrG* gene can in principle create a full length functional copy of the *pyrG* gene if the cross-over junction is located downstream of the *pyr181* mutation present in our auxotrophic strain. This should result in the genomic *pyrG* locus containing one non-functional, truncated copy of the gene and one functional, full-length copy of the gene (Fig. 6). Likewise, homologous cross-over integration of plasmid pPyrG-FH upstream of the genomic *pyr181* mutation should result in both copies of the *pyrG* gene being nonfunctional, and growth will not occur. Cross-over recombination can also lead to cleavage of the DHJ in such a way that gene replacement occurs with the chromosomal *pyr* mutation. While this recombination event was not detected with plasmid pPyrG-FH, it did occur in half of the transformants obtained with the plasmid pPyrG-FH/Ldh. This is obviously not a desirable recombination event in this particular case, since the plasmid and DNA of interest is not integrated into the genome. Even with *Agrobacterium*-mediated transformation, 25% of the *Rhizopus* isolates showed similar gene replacement events (Michielse et al. 2004). It is also recognized that it might be possible that the segment of the *pyrG* gene that is missing in plasmid pPyrG-FH could be restored by gene conversion. The 3′-OH on the truncated *pyrG* gene, resulting from *Mfe*I digestion, could use the genomic copy of *pyrG* to synthesize new DNA by BIR. However, it would be necessary for this newly synthesized DNA to reassociate with homologous plasmid template in order for conversion to occur.

This strategy of using a plasmid containing a truncated *pyrG* for integration has worked surprisingly well, with up to 100% of all isolates in this study having integrated the plasmid as originally hypothesized. While only a small number of isolates were tested, such targeted integration efficiency has never before been demonstrated in a Mucorales fungus. While *Agrobacterium*-mediated transformation of *Rhizopus* does represent a significant advance, it appears to still be vulnerable to the same difficulties associated with competition between different repair mechanisms (Van Attikum et al. 2001, 2003), which may explain why integration in *Rhizopus* occurred at an undefined locus (Michielse et al. 2004). Furthermore, integration of additional copies of pPyrG-FH seems to occur quite readily, probably due to NHEJ that takes place prior to integration, as seen in previous studies (Skory 2004). The transformation efficiency with this plasmid is considerably lower than with the plasmid pPyr225, which contains a functional *pyrG* gene. This is probably to be expected, since recombination in a specific manner is a prerequisite for restoring prototrophic growth. Unfortunately, we were unable to achieve any significant increase in transformation efficiency by modifying the biolistic protocol. It is likely that newer methods of introducing DNA will continue to raise the total number of transformants obtained with this procedure. However, we feel that this approach of using a truncated selectable marker is still a considerable improvement over current methods, considering that it results in such high integration efficiencies.

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